EXHIBIT 21

In some embodiments, the target need not actually be labeled if a means for detecting where interaction takes place is available. As described below, for a nucleic acid embodiment, such may be provided by an intercalating dye which intercalates only into double stranded segments, e.g., where interaction occurs. See, e.g., Sheldon et al. U.S. Pat. No. 4,582,789.

In many uses, the target sequence will be absolutely homogeneous, both with respect to the total sequence and with respect to the ends of each molecule. Homogeneity with respect to sequence is important to avoid ambiguity. It is preferable that the target sequences of interest not be contaminated with a significant amount of labeled contaminating sequences. The extent of allowable contamination will depend on the sensitivity of the detection system and the inherent signal to noise of the system. Homogeneous contamination sequences will be particularly disruptive of the sequencing procedure.

However, although the target polynucleotide must have a unique sequence, the target molecules need not have identical ends. In fact, the homogeneous target molecule preparation may be randomly sheared to increase the numerical number of molecules. Since the total information content remains the same, the shearing results only in a higher number of distinct sequences which may be labeled and bind to the probe. This fragmentation may give a vastly superior signal relative to a preparation of the target molecules having homogeneous ends.

The signal for the hybridization is likely to be dependent on the numerical frequency of the target-probe interactions. If a sequence is individually found on a larger number of separate molecules a better signal will result. In fact, shearing a h omogeneous preparation of the target may often be preferred before the labeling procedure is performed, thereby producing a large number of labeling groups associated with each subsequence.

C. Hybridization Conditions

The hybridization conditions between probe and target should be selected such that the specific recognition interaction, i.e., hybridization, of the two molecules is both sufficiently specific and sufficiently stable. See, e.g., Hames and Higgins (1985) *Nucleic Acid Hybridisation: A Practical Approach*, IRL Press, Oxford. These conditions will be dependent both on the specific sequence and often on the guanine and cytosine (GC) content of the complementary hybrid strands. The conditions may often be selected to be universally equally stable independent of the specific sequences involved. This typically will make use of a reagent such as an alkylammonium buffer. See, Wood et al. (1985) "Base Composition-independent Hybridization in Tetramethylammonium Chloride: A Method for Oligonucleotide Screening of Highly Complex Gene Libraries," *Proc. Natl. Acad. Sci. USA*, 82:1585–1588; and Krupov et al. (1989) "An Oligonucleotide Hybridization Approach to DNA Sequencing," *FEBS Letters*, 256:118–122; each of which is hereby incorporated herein by reference. An alkylammonium buffer tends to minimize differences in hybridization rate and stability due to GC content. By virtue of the fact that sequences then hybridize with approximately equal affinity and stability, there is relatively little bias in strength or kinetics of binding for particular sequences. Temperature and salt conditions along with other buffer parameters should be selected such that the kinetics of renaturation should be essentially independent of the specific target subsequence or oligonucleotide probe involved. In order to ensure this, the hybridization reactions will usually be performed in a single incubation of all the substrate matrices together exposed to the identical same target probe solution under the same conditions.

Alternatively, various substrates may be individually treated differently. Different substrates may be produced, each having reagents which bind to target subsequences with substantially identical stabilities and kinetics of hybridization. For example, all of the high GC content probes could be synthesized on a single substrate which is treated accordingly. In this embodiment, the arylammonium buffers could be unnecessary. Each substrate is then treated in a manner such that the collection of substrates show essentially uniform binding and the hybridization data of target binding to the individual substrate matrix is combined with the data from other substrates to derive the necessary subsequence binding information. The hybridization conditions will usually be selected to be sufficiently specific such that the fidelity of base matching will be properly discriminated. Of course, control hybridizations should be included to determine the stringency and kinetics of hybridization.

D. Detection; VLSIPS™ Technology Scanning

The next step of the sequencing process by hybridization involves labeling of target polynucleotide molecules. A quickly and easily detectable signal is preferred. The VLSIPS™ Technology apparatus is designed to easily detect a fluorescent label, so fluorescent tagging of the target sequence is preferred. Other suitable labels include heavy metal labels, magnetic probes, chromogenic labels (e.g., phosphorescent labels, dyes, and fluorophores) spectroscopic labels, enzyme linked labels, radioactive labels, and labeled binding proteins. Additional labels are described in U.S. Pat. No. 4,366,241, which is incorporated herein by reference.

The detection methods used to determine where hybridization has taken place will typically depend upon the label selected above. Thus, for a fluorescent label a fluorescent detection step will typically be used. Pirrung et al. (1992) U.S. Pat. No. 5,143,854 and Ser. No. 07/624,120, now abandoned, describe apparatus and mechanisms for scanning a substrate matrix using fluorescence detection, but a similar apparatus is adaptable for other optically detectable labels.

The detection method provides a positional localization of the region where hybridization has taken place. However, the position is correlated with the specific sequence of the probe since the probe has specifically been attached or synthesized at a defined substrate matrix position. Having collected all of the data indicating the subsequences present in the target sequence, this data may be aligned by overlap to reconstruct the entire sequence of the target, as illustrated above.

It is also possible to dispense with actual labeling if some means for detecting the positions of interaction between the sequence specific reagent and the target molecule are available. This may take the form of an additional reagent which can indicate the sites either of interaction, or the sites of lack of interaction, e.g., a negative label. For the nucleic acid embodiments, locations of double strand interaction may be detected by the incorporation of intercalating dyes, or other reagents such as antibody or other reagents that recognize helix formation, see, e.g., Sheldon, et al. (1986) U.S. Pat. No. 4,582,789, which is hereby incorporated herein by reference.

E. Analysis

Although the reconstruction can be performed manually as illustrated above, a computer program will typically be used to perform the overlap analysis. A program may be written and run on any of a large number of different

computer hardware systems. The variety of operating systems and languages useable will be recognized by a computer software engineer. Various different languages may be used, e.g., BASIC; C; PASCAL; etc. A simple flow chart of data analysis is illustrated in FIG. 1.

F. Substrate Reuse

Finally, after a particular sequence has been hybridized and the pattern of hybridization analyzed, the matrix substrate should be reusable and readily prepared for exposure to a second or subsequent target polynucleotides. In order to do so, the hybrid duplexes are disrupted and the matrix treated in a way which removes all traces of the original target. The matrix may be treated with various detergents or solvents to which the substrate, the oligonucleotide probes, and the linkages to the substrate are inert. This treatment may include an elevated temperature treatment, treatment with organic or inorganic solvents, modifications in pH, and other means for disrupting specific interaction. Thereafter, a second target may actually be applied to the recycled matrix and analyzed as before.

G. Non-Polynucleotide Aspects

Although the sequencing, fingerprinting, and mapping functions will make use of the natural sequence recognition property of complementary nucleotide sequences, the non-polynucleotide sequences typically require other sequence recognition reagents. These reagents will take the form, typically, of proteins exhibiting binding specificity, e.g., enzyme binding sites or antibody binding sites.

Enzyme binding sites may be derived from promoter proteins, restriction enzymes, and the like. See, e.g., Stryer, L. (1988) *Biochemistry*, W.H. Freeman, Palo Alto. Antibodies will typically be produced using standard procedures, see, e.g., Harlow and Lane (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Press, New York; and Goding (1986) *Monoclonal Antibodies: Principles and Practice*, (2d Ed.) Academic Press, San Diego.

Typically, an antigen, or collection of antigens are presented to an immune system. This may take the form of synthesized short polymers produced by the VLSIPS technology, or by the other synthetic means, or from isolation of natural products. For example, antigen for the polypeptides may be made by the VLSIPS technology, by standard peptide synthesis, by isolation of natural proteins with or without degradation to shorter segments, or by expression of a collection of short nucleic acids of random or defined sequences. See, e.g., Tuerk and Gold (1990) *Science* 249:505–510, for generation of a collection of randomly mutagenized oligonucleotides useful for expression.

The antigen or collection is presented to an appropriate immune system, e.g., to a whole animal as in a standard immunization protocol, or to a collection of immune cells or equivalent. In particular, see Ward et al. (1989) *Nature* 341:544–546; and Huse et al. (1989) *Science* 246:1275–1281, each of which is hereby incorporated herein by reference.

A large diversity of antibodies will be generated, some of which have specificities for the desired sequences. Antibodies may be purified having the desired sequence specificities by isolating the cells producing them. For example, a VLSIPS substrate with the desired antigens synthesized thereon may be used to isolate cells with cell surface reagents which recognize the antigens. The VLSIPS substrate may be used as an affinity reagent to select and recover the appropriate cells. Antibodies from those cells may be attached to a substrate using the caged biotin methodology, or by attaching a targeting molecule, e.g., an oligonucleotide. Alternatively, the supernatants from antibody producing cells can be easily assayed using a VLSIPS substrate to identify the cells producing the appropriate antibodies.

Although cells may be isolated, specific antibody molecules which perform the sequence recognition will also be sufficient. Preferably populations of antibody with a known specificity can be isolated. Supernatants from a large population of producing cells may be passed over a VLSIPS substrate to bind to the desired antigens attached to the substrate. When a sufficient density of antibody molecules are attached, they may be removed by an automated process, preferably as antibody populations exhibiting specificity of binding.

In one particular embodiment, a VLSIPS substrate, e.g., with a large plurality of fingerprint antigens attached thereto, is used to isolate antibodies from a supernatant of a population of cells producing antibodies to the antigens. Using the substrate as an affinity reagent, the antibodies will attach to the appropriate positionally defined antigens. The antibodies may be carefully removed therefrom, preferably by an automated system which retains their homogeneous specificities. The isolated antibodies can be attached to a new substrate in a positionally defined matrix pattern.

In a further embodiment, these spatially separated antibodies may be isolated using a specific targeting method for isolation. In this embodiment, a linker molecule which attaches to a particular portion of the antibody, preferably away from the binding site, can be attached to the antibodies. Various reagents will be used, including staphylococcus protein A or antibodies which bind to domains remote from the binding site. Alternatively, the antibodies in the population, before affinity purification, may be derivatized with an appropriate reagent compatible with new VLSIPS synthesis. A preferred reagent is a nucleotide which can serve as a linker to synthetic VLSIPS steps for synthesizing a specific sequence thereon. Then, by successive VLSIPS cycles, each of the antibodies attached to the defined antigen regions can have a defined oligonucleotide synthesized thereon and corresponding in area to the region of the substrate having each antigen attached. These defined oligonucleotides will be useful as targeting reagents to attach those antibodies possessing the same target sequence specificity at defined positions on a new substrate, by virtue of having bound to the antigen region, to a new VLSIPS substrate having the complementary target oligonucleotides positionally located on it. In this fashion, a VLSIPS substrate having the desired antigens attached thereto can be used to generate a second VLSIPS substrate with positionally defined reagents which recognize those antigens.

The selected antigens will typically be selected to be those which define particular functionalities or properties, so as to be useful for fingerprinting and other uses. They will also be useful for mapping and sequencing embodiments.

IV. FINGERPRINTING

A. General

Many of the procedures and techniques used in the polynucleotide sequencing section are also appropriate for fingerprinting applications. See, e.g., Poustka, et al. (1986) *Cold Spring Harbor Symposia on Quant. Biol.*, vol. LI, 131–139, Cold Spring Harbor Press, New York; which is hereby incorporated herein by reference. The fingerprinting method provided herein is based, in part, upon the ability to positionally localize a large number of different specific probes onto a single substrate. This high density matrix pattern provides the ability to screen for, or detect, a very large number of different sequences simultaneously. In fact, depending upon the hybridization conditions, fingerprinting

to the resolution of virtually absolute matching of sequence is possible thereby approaching an absolute sequencing embodiment. And the sequencing embodiment is very useful in identifying the probes useful in further fingerprinting uses. For example, characteristic features of genetic sequences will be identified as being diagnostic of the entire sequence. However, in most embodiments, longer probe and target will be used, and for which slight mismatching may not need to be resolved.

B. Preparation of Substrate Matrix

A collection of specific probes may be produced by either of the methods described above in the section on sequencing. Specific oligonucleotide probes of desired lengths may be individually synthesized on a standard oligonucleotide synthesizer. The length of these probes is limited only by the ability of the synthesizer to continue to accurately synthesize a molecule. Oligonucleotides or sequence fragments may also be isolated from natural sources. Biological amplification methods may be coupled with synthetic synthesizing procedures such as, e.g., polymerase chain reaction.

In one embodiment, the individually isolated probes may be attached to the matrix at defined positions. These probe reagents may be attached by an automated process making use of the caged biotin methodology described in Ser. No. 07/612,671, or using photochemical reagents, see, e.g., Dattagupta et al. (1985) U.S. Pat. No. 4,542,102 and (1987) U.S. Pat. No. 4,713,326. Each individually purified reagent can be attached individually at specific locations on a substrate.

In another embodiment, the VLSIPS synthesizing technique may be used to synthesize the desired probes at specific positions on a substrate. The probes may be synthesized by successively adding appropriate monomer subunits, e.g., nucleotides, to generate the desired sequences.

In another embodiment, a relatively short specific oligonucleotide is used which serves as a targeting reagent for positionally directing the sequence recognition reagent. For example, the sequence specific reagents having a separate additional sequence recognition segment (usually of a different polymer from the target sequence) can be directed to target oligonucleotides attached to the substrate. By use of non-natural targeting reagents, e.g., unusual nucleotide analogues which pair with other unnatural nucleotide analogues and which do not interfere with natural nucleotide interactions, the natural and non-natural portions can coexist on the same molecule without interfering with their individual functionalities. This can combine both a synthetic and biological production system analogous to the technique for targeting monoclonal antibodies to locations on a VLSIPS substrate at defined positions. Unnatural optical isomers of nucleotides may be useful unnatural reagents subject to similar chemistry, but incapable of interfering with the natural biological polymers. See also, Ser. No. 07/626,730, which is hereby incorporated herein by reference.

After the separate substrate attached reagents are attached to the targeting segment, the two are crosslinked, thereby permanently attaching them to the substrate. Suitable crosslinking reagents are known, see, e.g., Dattagupta et al. (1985) U.S. Pat. No. 4,542,102 and (1987) "Coupling of nucleic acids to solid support by photochemical methods," U.S. Pat. No. 4,713,326, each of which is hereby incorporated herein by reference. Similar linkages for attachment of proteins to a solid substrate are provided, e.g., in Merrifield (1986) *Science* 232:341–347, which is hereby incorporated herein by reference.

C. Labeling Target Nucleotides

The labeling procedures used in the sequencing embodiments will also be applicable in the fingerprinting embodiments. However, since the fingerprinting embodiments often will involve relatively large target molecules and relatively short oligonucleotide probes, the amount of signal necessary to incorporate into the target sequence may be less critical than in the sequencing applications. For example, a relatively long target with a relatively small number of labels per molecule may be easily amplified or detected because of the relatively large target molecule size.

In various embodiments, it may be desired to cleave the target into smaller segments as in the sequencing embodiments. The labeling procedures and cleavage techniques described in the sequencing embodiments would usually also be applicable here.

D. Hybridization Conditions

The hybridization conditions used in fingerprinting embodiments will typically be less critical than for the sequencing embodiments. The reason is that the amount of mismatching which may be useful in providing the fingerprinting information would typically be far greater than that necessary in sequencing uses. For example, Southern hybridizations do not typically distinguish between slightly mismatched sequences. Under these circumstances, important and valuable information may be arrived at with less stringent hybridization conditions while providing valuable fingerprinting information. However, since the entire substrate is typically exposed to the target molecule at one time, the binding affinity of the probes should usually be of approximately comparable levels. For this reason, if oligonucleotide probes are being used, their lengths should be approximately comparable and will be selected to hybridize under conditions which are common for most of the probes on the substrate. Much as in a Southern hybridization, the target and oligonucleotide probes are of lengths typically greater than about 25 nucleotides. Under appropriate hybridization conditions, e.g., typically higher salt and lower temperature, the probes will hybridize irrespective of imperfect complementarity. In fact, with probes of greater than, e.g., about fifty nucleotides, the difference in stability of different sized probes will be relatively minor.

Typically the fingerprinting is merely for probing similarity or homology. Thus, the stringency of hybridization can usually be decreased to fairly low levels. See, e.g., Wetmur and Davidson (1968) "Kinetics of Renaturation of DNA," *J. Mol. Biol.*, 31:349–370; and Kanehisa, M. (1984) *Nuc. Acids Res.*, 12:203–213.

E. Detection; VLSIPS™ Technology Scanning

Detection methods will be selected which are appropriate for the selected label. The scanning device need not necessarily be digitized or placed into a specific digital database, though such would most likely be done. For example, the analysis in fingerprinting could be photographic. Where a standardized fingerprint substrate matrix is used, the pattern of hybridizations may be spatially unique and may be compared photographically. In this manner, each sample may have a characteristic pattern of interactions and the likelihood of identical patterns will preferably be such low frequency that the fingerprint pattern indeed becomes a characteristic pattern virtually as unique as an individuals fingertip fingerprint. With a standardized substrate, every individual could be, in theory, uniquely identifiable on the basis of the pattern of hybridizing to the substrate.

Of course, the VLSIPS™ Technology scanning apparatus may also be useful to generate a digitized version of the fingerprint pattern. In this way, the identification pattern can

be provided in a linear string of digits. This sequence could also be used for a standardized identification system providing significant useful medical transferability of specific data. In one embodiment, the probes used are selected to be of sufficiently high resolution to measure the antigens of the major histo compatibility complex. It might even be possible to provide transplantation matching data in a linear stream of data. The fingerprinting data may provide a condensed version, or summary, of the linear genetic data, or any other information data base.

F. Analysis

The analysis of the fingerprint will often be much simpler than a total sequence determination. However, there may be particular types of analysis which will be substantially simplified by a selected group of probes. For example, probes which exhibit particular populational heterogeneity may be selected. In this way, analysis may be simplified and practical utility enhanced merely by careful selection of the specific probes and a careful matrix layout of those probes.

G. Substrate Reuse

As with the sequencing application, the fingerprinting usages may also take advantage of the reusability of the substrate. In this way, the interactions can be disrupted, the substrate treated, and the renewed substrate is equivalent to an unused substrate.

H. Non-polynucleotide Aspects

Besides polynucleotide applications, the fingerprinting analysis may be applied to other polymers, especially polypeptides, carbohydrates, and other polymers, both organic and inorganic. Besides using the fingerprinting method for analyzing a particular polymer, the fingerprinting method may be used to characterize various samples. For example, a cell or population of cells may be tested for their expression of specific antigens or their mRNA sequence intent. For example, a T-cell may be classified by virtue of its combination of expressed surface antigens. With specific reagents which interact with these antigens, a cell or a population of cells or a lysed cell may be exposed to a VLSIPS substrate. The biological sample may be classified or characterized by analyzing the pattern of specific interaction.

This may be applicable to a cell or tissue type, to the messenger RNA population expressed by a cell to the genetic content of a cell, or to virtually any sample which can be classified and/or identified by its combination of specific molecular properties.

The ability to generate a high density means for screening the presence or absence of specific interactions allows for the possibility of screening for, if not saturating, all of a very large number of possible interactions. This is very powerful in providing the means for testing the combinations of molecular properties which can define a class of samples. For example, a species of organism may be characterized by its DNA sequences, e.g., a genetic fingerprint. By using a fingerprinting method, it may be determined that all members of that species are sufficiently similar in specific sequences that they can be easily identified as being within a particular group. Thus, newly defined classes may be resolved by their similarity in fingerprint patterns. Alternatively, a non-member of that group will fail to share those many identifying characteristics. However, since the technology allows testing of a very large number of specific interactions, it also provides the ability to more finely distinguish between closely related different cells or samples. This will have important applications in diagnosing viral, bacterial, and other pathological on nonpathological infections.

In particular, cell classification may be defined by any of a number of different properties. For example, a cell class may be defined by its DNA sequences contained therein. This allows species identification for parasitic or other infections. For example, the human cell is presumably genetically distinguishable from a monkey cell, but different human cells will share many genetic markers. At higher resolution, each individual human genome will exhibit unique sequences that can define it as a single individual.

Likewise, a developmental stage of a cell type may be definable by its pattern of expression of messenger RNA. For example, in particular stages of cells, high levels of ribosomal RNA are found whereas relatively low levels of other types of messenger RNAs may be found. The high resolution distinguishability provided by this fingerprinting method allows the distinction between cells which have relatively minor differences in its expressed mRNA population. Where a pattern is shown to be characteristic of a stage, a stage may be defined by that particular pattern of messenger RNA expression.

In a similar manner, the antigenic determinants found on a protein may very well define the cell class. For example, immunological T-cells are distinguishable from B-cells because, in part, the cell surface antigens on the cell types are distinguishable. Different T-cell subclasses can be also distinguished from one another by whether they contain particular T-cell antigens. The present invention provides the possibility for high resolution testing of many different interactions simultaneously, and the definition of new cell types will be possible.

The high resolution VLSIPS™ substrate may also be used as a very powerful diagnostic tool to test the combination of presence, of a plurality of different assays from a biological sample. For example, a cancerous condition may be indicated by a combination of various different properties found in the blood. For example, a cancerous condition may be indicated by a combination of expression of various soluble antigens found in the blood along with a high number of various cellular antigens found on lymphocytes and/or particular cell degradation products. With a substrate as provided herein, a large number of different features can be simultaneously performed on a biological sample. In fact, the high resolution of the test will allow more complete characterization of parameters which define particular diseases. Thus, the power of diagnostic tests may be limited by the extent of statistical correlation with a particular condition rather than with the number of antigens or interactions which are tested. The present invention provides the means to generate this large universe of possible reagents and the ability to actually accumulate that correlative data.

In another embodiment, a substrate as provided herein may be used for genetic screening. This would allow for simultaneous screening of thousands of genetic markers. As the density of the matrix is increased, many more molecules can be simultaneously tested. Genetic screening then becomes a simpler method as the present invention provides the ability to screen for thousands, tens of thousands, and hundreds of thousands, even millions of different possible genetic features. However, the number of high correlation genetic markers for conditions numbers only in the hundreds. Again, the possibility for screening a large number of sequences provides the opportunity for generating the data which can provide correlation between sequences and specific conditions or susceptibility. The present invention provides the means to generate extremely valuable correlations useful for the genetic detection of the causative mutation leading to medical conditions. In still another embodiment, the present invention would be applicable to distinguishing two individuals having identical genetic compositions. The antibody population within an individual is dependent both on genetic and historical factors. Each

individual experiences a unique exposure to various infectious agents, and the combined antibody expression is partly determined thereby. Thus, individuals may also be fingerprinted by their immunological content, either of actively expressed antibodies, or their immunological memory. Similar sorts of immunological and environmental histories may be useful for fingerprinting, perhaps in combination with other screening properties. In particular, the present invention may be useful for screening allergic reactions or susceptibilities, and a simple IgE specificity test may be useful in determining a spectrum of allergies.

With the definition of new classes of cells, a cell sorter will be used to purify them. Moreover, new markers for defining that class of cells will be identified. For example, where the class is defined by its RNA content, cells may be screened by antisense probes which detect the presence or absence of specific sequences therein. Alternatively, cell lysates may provide information useful in correlating intracellular properties with extracellular markers which indicate functional differences. Using standard cell sorter technology with a fluorescence or labeled antisense probe which recognizes the internal presence of the specific sequences of interest, the cell sorter will be able to isolate a relatively homogeneous population of cells possessing the particular marker. Using successive probes the sorting process should be able to select for cells having a combination of a large number of different markers.

In a non-polynucleotide embodiment, cells-may be defined by the presence of other markers. The markers may be carbohydrates, proteins, or other molecules. Thus, a substrate having particular specific reagents, e.g., antibodies, attached to it should be able to identify cells having particular patterns of marker expression. Of course, combinations of these made be utilized and a cell class may be defined by a combination of its expressed mRNA, its carbohydrate expression, its antigens, and other properties. This fingerprinting should be useful in determining the physiological state of a cell or population of cells.

Having defined a cell type whose function or properties are defined by the reagents attachable to a VLSIPS substrate, such as cellular antigens, these structural manifestations of function may be used to sort cells to generate a relatively homogeneous population of that class of cells. Standard cell sorter technology may be applied to purify such a population, see, e.g., Dangl, J. and Herzenberg (1982) "Selection of hybridomas and hybridoma variants using the fluorescence activated cell sorter," *J. Immunological Methods* 52:1–14; and Becton Dickinson, Fluorescence Activated Cell Sorter Division, San Jose, Calif., and Coulter Diagnostics, Hialeah, Fla.

With the fingerprinting method an identification means arises from mosaicism problems in an organism. A mosaic organism is one whose genetic content in different cells is significantly different. Various clonal populations should have similar genetic fingerprints, though different clonal populations may have different genetic contents. See, for example, Suzuki et al. *An Introduction to Genetic Analysis* (4th Ed.), Freeman and Co., New York, which is hereby incorporated herein by reference. However, this problem should be a relatively rare problem and could be more carefully evaluated with greater experience using the fingerprinting methods.

The invention will also find use in detecting changes, both genetic and antigenic, e.g., in a rapidly "evolving" protozoa infection, or similarly changing organism.

V. MAPPING

A. General

The use of the present invention for mapping parallels its use for fingerprinting and sequencing. Where a polymer is a linear molecule, the mapping provides the ability to locate particular segments along the length of the polymer. Branched polymers can be treated as a series of individual linear polymers. The mapping provides the ability to locate, in a relative sense, the order of various subsequences. This may be achieved using at least two different approaches.

The first approach is to take the large sequence and fragment it at specific points. The fragments are then ordered and attached to a solid substrate. For example, the clones resulting from a chromosome walking process may be individually attached to the substrate by methods, e.g., caged biotin techniques, indicated earlier. Segments of unknown map position will be exposed to the substrate and will hybridize to the segment which contains that particular sequence. This procedure allows the rapid determination of a number of different labeled segments, each mapping requiring only a single hybridization step once the substrate is generated. The substrate may be regenerated by removal of the interaction, and the next mapping segment applied.

In an alternative method, a plurality of subsequences can be attached to a substrate. Various short probes may be applied to determine which segments may contain particular overlaps. The theoretical basis and a description of this mapping procedure is contained in, e.g., Evans et al. 1989 "Physical Mapping of Complex Genomes by Cosmid Multiplex Analysis," *Proc. Natl. Acad. Sci. USA* 86:5030–5034, and other references cited above in the Section labeled "Overall Description." Using this approach, the details of the mapping embodiment are very similar to those used in the fingerprinting embodiment.

B. Preparation of Substrate Matrix

The substrate may be generated in either of the methods generally applicable in the sequencing and fingerprinting embodiments. The substrate may be made either synthetically, or by attaching otherwise purified probes or sequences to the matrix. The probes or sequences may be derived either from synthetic or biological means. As indicated above, the solid phase substrate synthetic methods may be utilized to generate a matrix with positionally defined sequences. In the mapping embodiment, the importance of saturation of all possible subsequences of a preselected length is far less important than in the sequencing embodiment, but the length of the probes used may be desired to be much longer. The processes for making a substrate which has longer oligonucleotide probes should not be significantly different from those described for the sequencing embodiments, but the optimization parameters may be modified to comply with the mapping needs.

C. Labeling

The labeling methods will be similar to those applicable in sequencing and fingerprinting embodiments. Again, it may be desirable to fragment the target sequences.

D. Hybridization/Specific Interaction

The specificity of interaction between-the targets and probe would typically be closer to those used for fingerprinting embodiments, where homology is more important than absolute distinguishability of high fidelity complementary hybridization. Usually, the hybridization conditions will be such that merely homologous segments will interact and provide a positive signal. Much like the fingerprinting embodiment, it may be useful to measure the extent of homology by successive incubations at higher stringency conditions. Or, a plurality of different probes, each having various levels of homology may be used. In either way, the spectrum of homologies can be measured.

Where non-nucleic acid hybridization is involved, the specific interactions may also be compared in a fingerprint-like manner. The specific reagents may have less specificity, e.g., monoclonal antibodies which recognize a broader spectrum of sequences may be utilized relative to a sequencing embodiment. Again, the specificity of interaction may be measured under various conditions of increasing stringency

to determine the spectrum of matching across the specific probes selected, or a number of different stringency reagents may be included to indicate the binding affinity.

E. Detection

The detection methods used in the mapping procedure will be virtually identical to those used in the fingerprinting embodiment. The detection methods will be selected in combination with the labeling methods.

F. Analysis

The analysis of the data in a mapping embodiment will typically be somewhat different from that in fingerprinting.

The fingerprinting embodiment will test for the presence or absence of specific or homologous segments. However, in the mapping embodiment, the existence of an interaction is coupled with some indication of the location of the interaction. The interaction is mapped in some manner to the physical polymer sequence. Some means for determining the relative positions of different probes is performed. This may be achieved by synthesis of the substrate in pattern, or may result from analysis of sequences after they have been attached to the substrate.

For example, the probes may be randomly positioned at various locations on the substrate. However, the relative positions of the various reagents in the original polymer may be determined by using short fragments, e.g., individually, as target molecules which determine the proximity of different probes. By an automated system of testing each different short fragment of the original polymer, coupled with proper analysis, it will be possible to determine which probes are adjacent one another on the original target sequence and correlate that with positions on the matrix. In this way, the matrix is useful for determining the relative locations of various new segments in the original target molecule. This sort of analysis is described in Evans, and the related references described above.

G. Substrate Reuse

The substrate should be reusable in the manner described in the fingerprinting section. The substrate is renewed by removal of the specific interactions and is washed and prepared for successive cycles of exposure to new target sequences.

H. Non-polynucleotide Aspects

The mapping procedure may be used on other molecules than polynucleotides. Although hybridization is one type of specific interaction which is clearly useful for use in this mapping embodiment, antibody reagents may also be very useful.

In the same way that polypeptide sequencing or other polymers may be sequenced by the reagents and techniques described in the sequencing section and fingerprinting section, the mapping embodiment may also be used similarly.

In another form of mapping, as described above in the fingerprinting section, the developmental map of a cell or biological system may be measured using fingerprinting type technology. Thus, the mapping may be along a temporal dimension rather than along a polymer dimension. The mapping or fingerprinting embodiments may also be used in determining the genetic rearrangements which may be genetically important, as in lymphocyte and B-cell development. In another example, various rearrangements or chromosomal dislocations may be tested by either the fingerprinting or mapping methods. These techniques are similar in many respects and the fingerprinting and mapping embodiments may overlap in many respects.

VI. ADDITIONAL SCREENING AND APPLICATIONS

A. Specific Interactions

As originally indicated in the parent filing of VLSIPS™ Technology, the production of a high density plurality of spatially segregated polymers provides the ability to generate a very large universe or repertoire of individually. and distinct sequence possibilities. As indicated above, particular oligonucleotides may be synthesized in automated fashion at specific locations on a matrix. In fact, these oligonucleotides may be used to direct other molecules to specific locations by linking specific oligonucleotides to other reagents which are in batch exposed to the matrix and hybridized in a complementary fashion to only those locations where the complementary oligonucleotide has been synthesized on the matrix. This allows for spatially attaching a plurality of different reagents onto the matrix instead of individually attaching each separate reagent at each specific location. Although the caged biotin method allows automated attachment, the speed of the caged biotin attachment process is relatively slow and requires a separate reaction for each reagent being attached. By use of the oligonucleotide method, the specificity of position can be done in an automated and parallel fashion. As each reagent is produced, instead of directly attaching each reagent at each desired position, the reagent may be attached to a specific desired complementary oligonucleotide which will ultimately be specifically directed toward locations on the matrix having a complementary oligonucleotide attached thereat.

In addition, the technology allows screening for specificity of interaction with particular reagents. For example, the oligonucleotide sequence specificity of binding of a potential reagent may be tested by presenting to the reagent all of the possible subsequences available for binding.

Although secondary or higher order sequence specific features might not be easily screenable using this technology, it does provide a convenient, simple, quick, and thorough screen of interactions between a reagent and its target recognition sequences. See, e.g., Pfeifer et al. (1989) *Science* 246:810–812.

For example, the interaction of a promoter protein with its target binding sequence may be tested for many different, or all, possible binding sequences. By testing the strength of interactions under various different conditions, the interaction of the promoter protein with each of the different potential binding sites may be analyzed. The spectrum of strength of interactions with each different potential binding site may provide significant insight into the types of features which are important in determining specificity.

An additional example of a sequence specific interaction between reagents is the testing of binding of a double stranded nucleic acid structure with a single stranded oligonucleotide. Often, a triple stranded structure is produced which has significant aspects of sequence specificity. Testing of such interactions with either sequences comprising only natural nucleotides, or perhaps the testing of nucleotide analogs may be very important in screening for particularly useful diagnostic or therapeutic reagents. See, e.g., Häner and Dervan (1990) *Biochemistry* 29:9761–6765, and references therein.

B. Sequence Comparisons

Once a gene is sequenced, the present invention provides a means to compare alleles or related sequences to locate and identify differences from the control sequence.

This would be extremely useful in further analysis of genetic variability at a specific gene locus.

C. Categorizations

As indicated above in the fingerprinting and mapping embodiments, the present invention is also useful in defining specific stages in the temporal sequence of cells, e.g., development, and the resulting tissues within an organism. For example, the developmental stage of a cell, or population of cells, can be dependent upon the expression of particular messenger RNAs or cellular antigens. The screening procedures provided allow for high resolution definition of new classes of cells. In addition, the temporal develop-

ment of particular cells will be characterized by the presence or expression of various mRNAs. Means to simultaneously screen a plurality or very large number of different sequences are provided. The combination of different markers made available dramatically increases the ability to distinguish fairly closely related cell types. Other markers may be combined with markers and methods made available herein to define new classifications of biological samples, e.g., based upon new combinations of markers.

The presence or absence of particular marker sequences will be used to define temporal developmental stages.

Once the stages are defined, fairly simple methods can be applied to actually purify those particular cells. For example, antisense probes or recognition reagents may be used with a cell sorter to select those cells containing or expressing the critical markers. Alternatively, the expression of those sequences may result in specific antigens which may also be used in defining cell classes and sorting those cells away from others. In this way, for example, it should be possible to select a class of omnipotent immune system cells which are able to completely regenerate a human immune system.

Based upon the cellular classes defined by the parameters made available by this technology, purified classes of cells having identifiable differences, structural or functional, are made available.

In an alternative embodiment, a plurality of antigens or specific binding proteins attached to the substrate may be used to define particular cell types. For example, subclasses of T-cells are defined, in part, by the combination of expressed cell surface antigens. The present invention allows for the simultaneous screening of a large plurality of different antigens together. Thus, higher resolution classification of different T-cell subclasses becomes possible and, with the definitions and functional differences which correlate with those antigenic or other parameters, the ability to purify those cell types becomes available. This is applicable not only to T-cells, but also to lymphocyte cells, or even to freely circulating cells. Many of the cells for which this would be most useful will be immobile cells found in particular tissues or organs. Tumor cells will be diagnosed or detected using these fingerprinting techniques. Coupled with a temporal change in structure, developmental classes may also be selected and defined using these technologies. The present invention also provides the ability not only to define new classes of cells based upon functional or structural differences, but it also provides the ability to select or purify populations of cells which share these particular properties. Standard cell sorting procedures using antibody markers may be used to detect extracellular features. Intracellular features would also be detectable by introducing the label reagents into the cell. In particular, antisense DNA or RNA molecules may be introduced into a cell to detect RNA sequences therein. See, e.g., Weintraub (1990) *Scientific American* 262:40–46.

D. Statistical Correlations

In an additional embodiment, the present invention also allows for the high resolution correlation of medical conditions with various different markers. For example, the presently available technology, when applied to amniocentesis or other genetic screening methods, typically screens for tens of different markers at most. The present invention allows simultaneous screening for tens, hundreds, thousands, tens of thousands, hundreds of thousands, and even millions of different genetic sequences. Thus, applying the fingerprinting methods of the present invention to a sufficiently large population allows detailed statistical analysis to be made, thereby correlating particular medical conditions with particular markers, typically antigenic or genetic. Tumor specific antigens will be identified using the present invention.

Various medical conditions may be correlated against an enormous data base of the sequences within an individual. Genetic propensities and correlations then become available and high resolution genetic predictability and correlation become much more easily performed. With the enormous data base, the reliability of the predictions is also better tested. Particular markers which are partially diagnostic of particular medical conditions or medical susceptibilities will be identified and provide direction in further studies and more careful analysis of the markers involved. Of course, as indicated above in the sequencing embodiment, the present invention will find much use in intense sequencing projects. For example, sequencing of the entire human genome in the human genome project will be greatly simplified and enabled by the present invention.

VI. FORMATION OF SUBSTRATE

The substrate is provided with a pattern of specific reagents which are positionally localized on the surface of the substrate. This matrix of positions is defined by the automated system which produces the substrate. The instrument will typically be one similar to that described in Pirrung et al. (1992) U.S. Pat. No. 5,143,854, and Ser. No. 07/624,120, now abandoned. The instrumentation described therein is directly applicable to the applications used here. In particular, the apparatus comprises a substrate, typically a silicon containing substrate, on which positions on the surface may be defined by a coordinate system of positions. These positions can be individually addressed or detected by the VLSIPS™ Technology apparatus.

Typically, the VLSIPS™ Technology apparatus uses optical methods used in semiconductor fabrication applications. In this way, masks may be used to photo-activate positions for attachment or synthesis of specific sequences on the substrate. These manipulations may be automated by the types of apparatus described in Pirrung et al. (1992) U.S. Pat. No. 5,143,854 and Ser. No. 07/624,120, now abandoned.

Selectively removable protecting groups allow creation of well defined areas of substrate surface having differing reactivities. Preferably, the protecting groups are selectively removed from the surface by applying a specific activator, such as electromagnetic radiation of a specific wavelength and intensity. More preferably, the specific activator exposes selected areas of surface to remove the protecting groups in the exposed areas.

Protecting groups of the present invention are used in conjunction with solid phase oligomer syntheses, such as peptide syntheses using natural or unnatural amino acids, nucleotide syntheses using deoxyribonucleic and ribonucleic acids, oligosaccharide syntheses, and the like. In addition to protecting the substrate surface from unwanted reaction, the protecting groups block a reactive end of the monomer to prevent self-polymerization. For instance, attachment of a protecting group to the amino terminus of an activated amino acid, such as the N-hydroxysuccinimide-activated ester of the amino acid prevents the amino terminus of one monomer from reacting with the activated ester portion of another during peptide synthesis.

Alternatively, the protecting group may be attached to the carboxyl group of an amino acid to prevent reaction at this site. Most protecting groups can be attached to either the amino or the carboxyl group of an amino acid, and the nature of the chemical synthesis will dictate which reactive group will require a protecting group. Analogously, attachment of a protecting group to the 5'-hydroxyl group of a nucleoside during synthesis using for example, phosphate-triester coupling chemistry, prevents the 5'-hydroxyl of one nucleoside from reacting with the 3'-activated phosphate-triester of another.

Regardless of the specific use, protecting groups are employed to protect a moiety on a molecule from reacting

with another reagent. Protecting groups of the present invention have the following characteristics: they prevent selected reagents from modifying the group to which they are attached; they are stable (that is, they remain attached) to the synthesis reaction conditions; they are removable under conditions that do not adversely affect the remaining structure; and once removed, do not react appreciably with the surface or surface-bound oligomer. The selection of a suitable protecting group will depend, of course, on the chemical nature of the monomer unit and oligomer, as well as the specific reagents they are to protect against.

In a preferred embodiment, the protecting groups will be photoactivatable. The properties and uses of photoreactive protecting compounds have been reviewed. See, McCray et al., *Ann. Rev. of Biophys. and Biophys. Chem.* (1989) 18:239–270, which is incorporated herein by reference. Preferably, the photosensitive protecting groups will be removable by radiation in the ultraviolet (UV) or visible portion of the electromagnetic spectrum. More preferably, the protecting groups will be removable by radiation in the near UV or visible portion of the spectrum. In some embodiments, however, activation may be performed by other methods such as localized heating, electron beam lithography, laser pumping, oxidation or reduction with microelectrodes, and the like. Sulfonyl compounds are suitable reactive groups for electron beam lithography. Oxidative or reductive removal is accomplished by exposure of the protecting group to an electric current source, preferably using microelectrodes directed to the predefined regions of the surface which are desired for activation. A more detailed description of these protective groups is provided in Ser. No. 07/624,120, now abandoned, which is hereby incorporated herein by reference.

The density of reagents attached to a silicon substrate may be varied by standard procedures. The surface area for attachment of reagents may be increased by modifying the silicon surface. For example, a matte surface may be machined or etched on the substrate to provide more sites for attachment of the particular reagents. Another way to increase the density of reagent binding sites is to increase the derivitization density of the silicon. Standard procedures for achieving this are described, below.

One method to control the derivatization density is to highly derivatize the substrate with photochemical groups at high density. The substrate is then photolyzed for various predetermined times, which photoactivate the groups at a measurable rate, and react them with a capping reagent. By this method, the density of linker groups may be modulated by using a desired time and intensity of photoactivation.

In many applications, the number of different sequences which may be provided may be limited by the density and the size of the substrate on which the matrix pattern is generated. In situations where the density is insufficiently high to allow the screening of the desired number of sequences, multiple substrates may be used to increase the number of sequences tested. Thus, the number of sequences tested may be increased by using a plurality of different substrates. Because the VLSIPS apparatus is almost fully automated, increasing the number of substrates does not lead to a significant increase in the number of manipulations which must be performed by humans. This again leads to greater reproducibility and speed in the handling of these multiple substrates.

A. Instrumentation

The concept of using VLSIPS™ Technology generally allows a pattern or a matrix of reagents to be generated. The procedure for making the pattern is performed by any of a number of different methods. An apparatus and instrumentation useful for generating a high density VLSIPS substrate is described in detail in Pirrung et al. (1992) U.S. Pat. No. 5,143,854 and Ser. No. 07/624,120, now abandoned.

B. Binary Masking

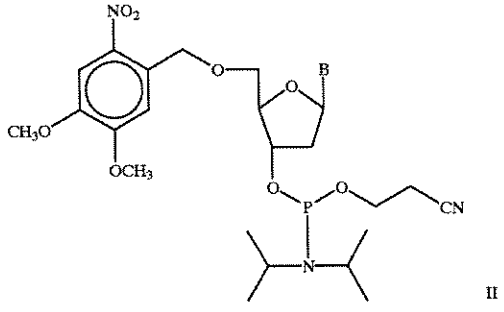
The details of the binary masking are described in an accompanying application filed simultaneously with this, Ser. No. 07/624,120, now abandoned, whose specification is incorporated herein by reference.

For example, the binary masking technique allows for producing a plurality of sequences based on the selection of either of two possibilities at any particular location. By a series of binary masking steps, the binary decision may be the determination, on a particular synthetic cycle, whether or not to add any particular one of the possible subunits. By treating various regions of the matrix pattern in parallel, the binary masking strategy provides the ability to carry out spatially addressable parallel synthesis.

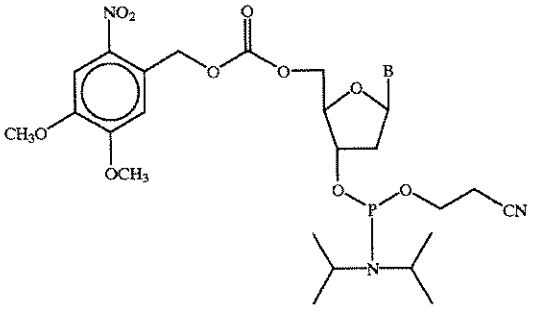
C. Synthetic Methods

The synthetic methods in making a substrate are described in the parent application, Pirrung et al. (1992) U.S. Pat. No. 5,143,854. The construction of the matrix pattern on the substrate will typically be generated by the use of photosensitive reagents. By use of photo-lithographic optical methods, particular segments of the substrate can be irradiated with light to activate or deactivate blocking agents, e.g., to protect or deprotect particular chemical groups. By an appropriate sequence of photo-exposure steps at appropriate times with appropriate masks and with appropriate reagents, the substrates can have known polymers synthesized at positionally defined regions on the substrate. Methods for synthesizing various substrates are described in Pirrung et al. (1992) U.S. Pat. No. 5,143,854 and Ser. No. 07/624,120, now abandoned. By a sequential series of these photo-exposure and reaction manipulations, a defined matrix pattern of known sequences may be generated, and is typically referred to as a VLSIPS™ Technology substrate. In the nucleic acid synthesis embodiment, nucleosides used in the synthesis of DNA by photolytic methods will typically be one of the two forms shown below:

I

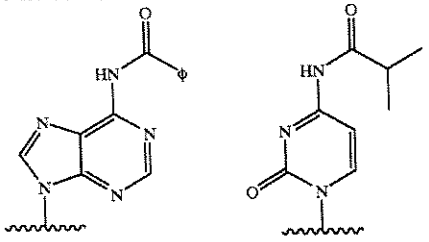


II



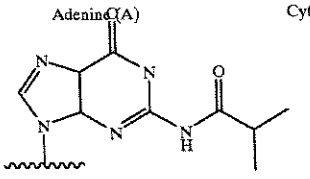
B=Adenine, Cytosine, Guanine, or Thymine

In I, the photolabile group at the 5' position is abbreviated NV (nitroveratryl) and in II, the group is abbreviated NVOC (nitroveratryl oxycarbonyl). Although not shown in FIG. C, the bases (adenine, cytosine, and guanine) contain exocyclic NH_2 groups which must be protected during DNA synthesis. Thymine contains no exocyclic NH_2 and therefore requires no protection. The standard protecting groups for these amines are shown below:



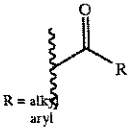
Adenine (A)

Cytosine (C)



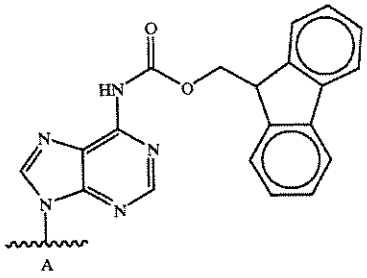
Guanine (G)

Other amides of the general formula

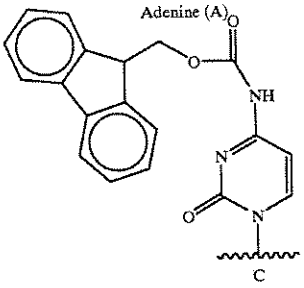


where R may be alkyl or aryl have been used.

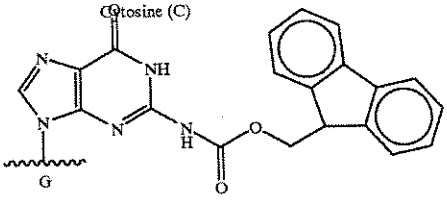
Another type of protecting group FMOC (9-fluorenyl methoxycarbonyl) is currently being used to protect the exocyclic amines of the three bases:



A



C

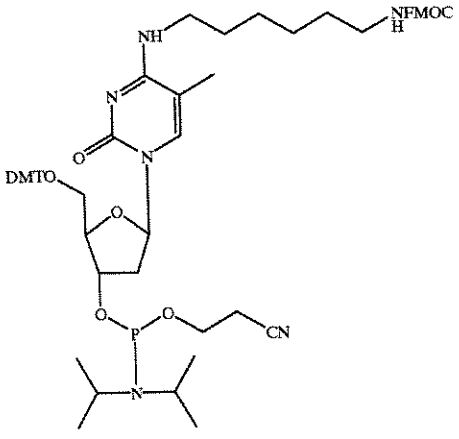


Guanine (G)

The advantage of the FMOC group is that it is removed under mild conditions (dilute organic bases) and can be used for all three bases. The amide protecting groups require more harsh conditions to be removed (NH_3/MeOH with heat).

Nucleosides used as 5'-OH probes, useful in verifying correct VLSIPS synthetic function, include, for example, the following:

III



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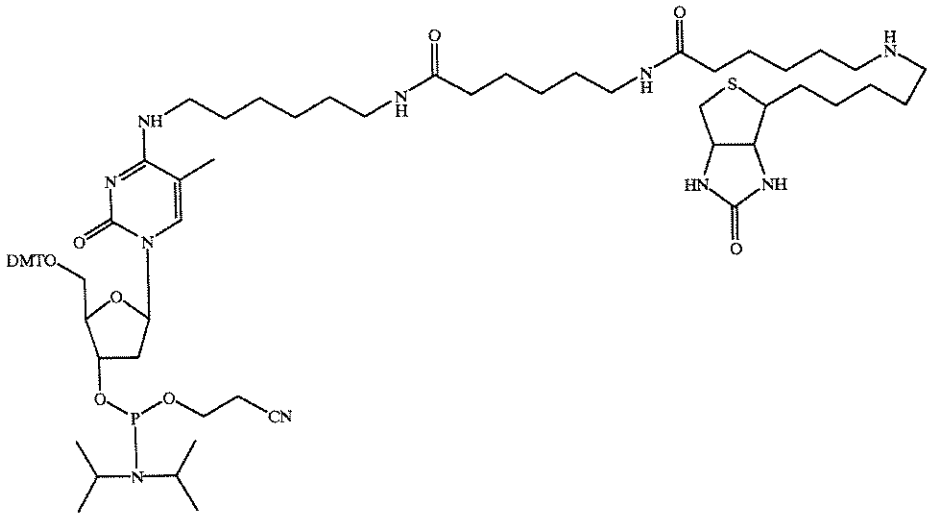
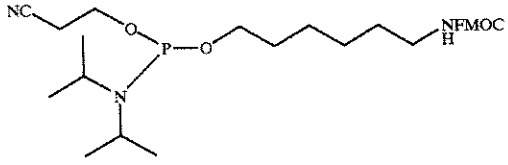
IV

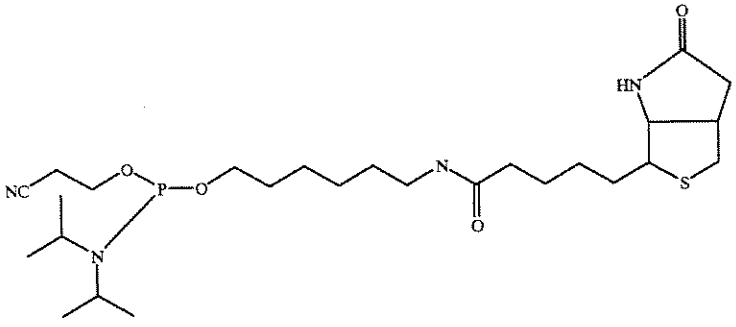


These compounds are used to detect where on a substrate photolysis has occurred by the attachment of either III or V to the newly generated 5'-OH. In the case of III, after the phosphate attachment is made, the substrate is treated with a dilute base to remove the FMOC group. The resulting amine can be reacted with FITC and the substrate examined by fluorescence microscopy. This indicates the proper generation of a 5'-OH. In the case of compound IV, after the phosphate attachment is made, the substrate is treated with FITC labeled streptavidin and the substrate again may be examined by fluorescence microscopy. Other probes, although not nucleoside based, have included the following:

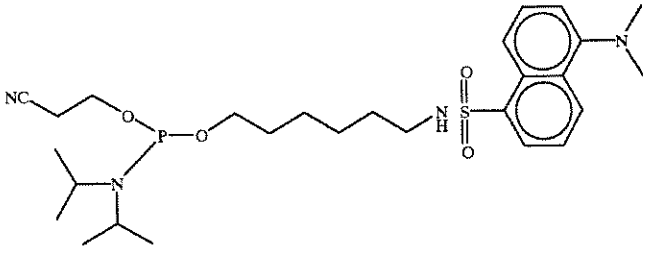
V



VI



VII



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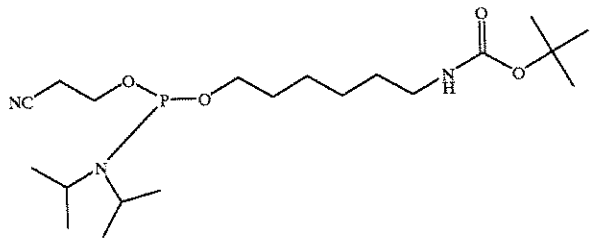
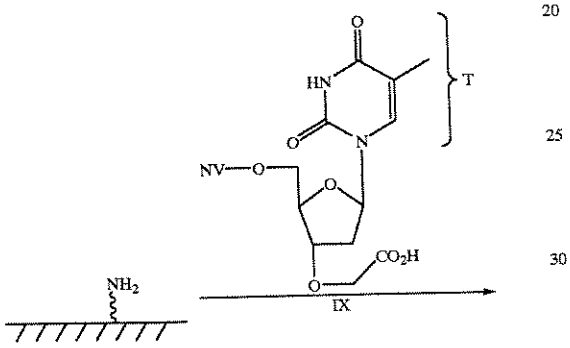
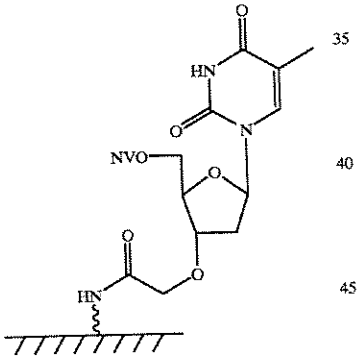


VIII

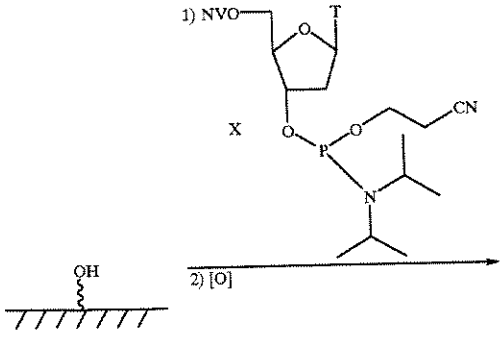
The method of attachment of the first nucleoside to the surface of the substrate depends on the functionality of the groups at the substrate surface. If the surface is amine functionalized, an amide bond is made (see example below).



IX



If the surface is hydroxy functionalized, a phosphate bond is made (see example below):



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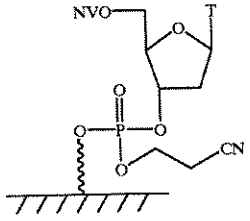
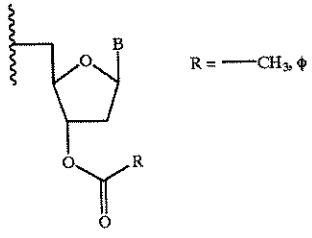


In both cases, the thymidine example is illustrated, but any one of the four phosphoramidite activated nucleosides can be used in the first step.

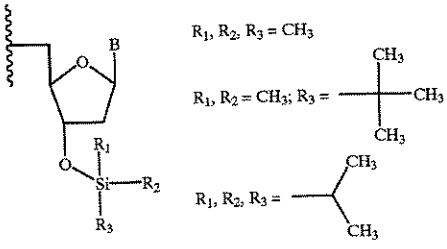
Photolysis of the photolabile group NV or NVOC on the 5' positions of the nucleosides is carried out at ~362 nm with an intensity of 14 mW/cm^2 for 10 minutes with the substrate side (side containing the photolabile group) immersed in dioxane. After the coupling of the next nucleoside is complete, the photolysis is repeated followed by another coupling until the desired oligomer is obtained.

One of the most common 3'-O-protecting groups is the ester, in particular the acetate:



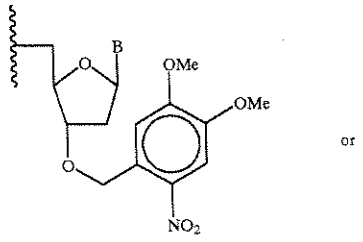
The groups can be removed by mild base treatment 0.1N NaOH/MeOH or K_2CO_3/H_2O/MeOH.

Another group used most often is the silyl ether:

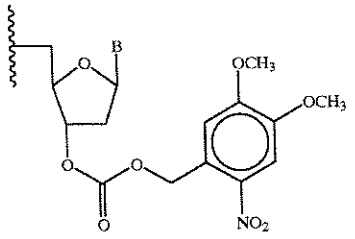


These groups can be removed by neutral conditions using 1 M tetra-n-butylammonium fluoride in THF or under acid conditions.

With respect to photodeprotection, the nitroveratryl group could also be used to protect the 3'-position.

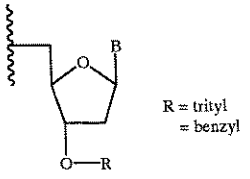


or



Here, light (photolysis) would be used to remove these protecting groups.

A variety of ethers can also be used in the protection of the 3'-O-position:



R = trityl
= benzyl

Removal of these groups usually involves acid or catalytic methods.

Note that corresponding linkages and photoblocked amino acids are described in detail in Ser. No. 07/624,120' now abandoned, which is hereby incorporated herein by reference.

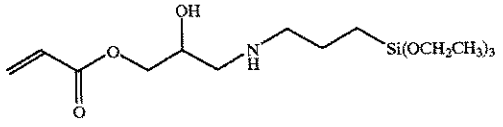
Although the specificity of interactions at particular locations will usually be homogeneous due to a homogeneous polymer being synthesized at each defined location, for certain purposes, it may be useful to have mixed polymers with a commensurate mixed collection of interactions occurring at specific defined locations, or degeneracy reducing analogues, which have been discussed above and show broad specificity in binding. Then, a positive interaction signal may result from any of a number of sequences contained therein.

As an alternative method of generating a matrix pattern on a substrate, preformed polymers may be individually attached at particular sites on the substrate. This may be performed by individually attaching reagents one at a time to specific positions on the matrix, a process which may be automated. See, e.g., Ser. No. 07/435,316, now abandoned, and Barrett et al. (1993) U.S. Pat. No. 5,252,743. Another way of generating a positionally defined matrix pattern on a substrate is to have individually specific reagents which interact with each specific position on the substrate. For example, oligonucleotides may be synthesized at defined locations on the substrate. Then the substrate would have on its surface a plurality of regions having homogeneous oligonucleotides attached at each position.

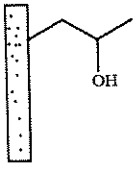
In particular, at least four different substrate preparation procedures are available for treating a substrate surface. They are the standard VLSIPS™ Technology method, polymeric substrates, Durapore™, and synthetic beads or fibers. The treatment labeled "standard VLSIPS™ Technology" method is described in Ser. No. 07/624,120, now abandoned, and involves applying amino-propyltriethoxysilane to a glass surface.

The polymeric substrate approach involves either of two ways of generating a polymeric substrate. The first uses a high concentration of aminopropyltriethoxysilane (2–20%) in an aqueous ethanol solution (95%). This allows the silane compound to polymerize both in solution and on the substrate surface, which provides a high density of amines on the surface of the glass. This density is contrasted with the standard VLSIPS method. This polymeric method allows for the deposition on the substrate surface of a monolayer due to the anhydrous method used with the aforementioned silane.

The second polymeric method involves either the coating or covalent binding of an appropriate acrylic acid polymer onto the substrate surface. In particular, e.g., in DNA synthesis, a monomer such as a hydroxypropylacrylate is used to generate a high density of hydroxyl groups on the substrate surface, allowing for the formation of phosphate bonds. An example of such a compound is shown:



The method using a Durapore™ membrane (Millipore) consists of a polyvinylidine difluoride coating with crosslinked polyhydroxylpropyl acrylate [PVDF-HPA]:



Here the building up of, e.g., a DNA oligomer, can be started immediately since phosphate bonds to the surface can be accomplished in the first step with no need for modification. A nucleotide dimer (5'-C-T-3') has been successfully made on this substrate.

The fourth method utilizes synthetic beads or fibers. This would use another substrate, such as a teflon copolymer graft bead or fiber, which is covalently coated with an organic layer (hydrophilic) terminating in hydroxyl sites (commercially available from Molecular Biosystems, Inc.) This would offer the same advantage as the Durapore™ membrane, allowing for immediate phosphate linkages, but would give additional contour by the 3-dimensional growth of oligomers.

A matrix pattern of new reagents may be targeted to each specific oligonucleotide position by attaching a complementary oligonucleotide to which the substrate bound form is complementary. For instance, a number of regions may have homogeneous oligonucleotides synthesized at various locations. Oligonucleotide sequences complementary to each of these can be individually generated and linked to a particular

specific reagents. Often these specific reagents will be antibodies. As each of these is specific for finding its complementary oligonucleotide, each of the specific reagents will bind through the oligonucleotide to the appropriate matrix position. A single step having a combination of different specific reagents being attached specifically to a particular oligonucleotide will thereby bind to its complement at the defined matrix position. The oligonucleotides will typically then be covalently attached, using, e.g., an acridine dye, for photocrosslinking. Psoralen is a commonly used acridine dye for photocrosslinking purposes, see, e.g., Song et al. (1979) *Photochem. Photobiol.* 29:1177–1197; Cimino et al. (1985) *Ann. Rev. Biochem.* 54:1151–1193; Parsons (1980) *Photochem. Photobiol.* 32:813–821; and Dattagupta et al. (1985) U.S. Pat.

No. 4,542,102, and (1987) U.S. Pat. No. 4,713,326; each of which is hereby incorporated herein by reference. This method allows a single attachment manipulation to attach all of the specific reagents to the matrix at defined positions and results in the specific reagents being homogeneously located at defined positions. In many embodiments, the specific reagents will be antibodies.

In an alternative embodiment, antibody molecules may be used to specifically direct binding to defined positions on a substrate. The VLSIPS technology may be used to generate specific epitopes at each position on the substrate. Antibody molecules having specificity of interaction may be used to attach oligonucleotides, thereby avoiding the interference of internal polynucleotide sequences from binding to the substrate complementary oligonucleotides. In fact, the specificity of interaction for positional targeting may be achieved by use of nucleotide analogues which do not interact with the natural nucleotides. For example, other synthetic nucleotides have been made which undergo base pairing, thereby providing the specificity of targeting, but the synthetic nucleotides also do not interact with the natural biological nucleotides. Thus, synthetic oligonucleotides would be useful for attachment to biological nucleotides and specific targeting. Moreover, the VLSIPS synthetic processes would be useful in generating the VLSIPS substrate, and standard oligonucleotide synthesis could be applied, with minor modifications, to produce the complementary sequences which would be attached to other specific reagents.

D. Surface Immobilization

1. Caged Biotin

An alternative method of attaching reagents in a positionally defined matrix pattern is to use a caged biotin system. See Barrett et al. (1993) U.S. Pat. No. 5,252,743, which is hereby incorporated herein by reference, for additional details on the chemistry and application of caged biotin embodiments. In short, the caged biotin has a photosensitive blocking moiety which prevents the combination of avidin to biotin. At positions where the photo-lithographic process has removed the blocking group, high affinity biotin sites are generated. Thus, by a sequential series of photolithographic deblocking steps interspersed with exposure of those regions to appropriate biotin containing reagents, only those locations where the deblocking takes place will form an avidin-biotin interaction. Because the avidin-biotin binding is very tight, this will usually be virtually irreversible bindings

2. Crosslinked Interactions

The surface immobilization may also take place by photo crosslinking of defined oligonucleotides linked to specific reagents. After hybridization of the complementary oligonucleotides, the oligonucleotides may be crosslinked by a reagent by psoralen or another similar type of acridine dye. Other useful cross linking reagents are described in Dattagupta et al. (1985) U.S. Pat. No. 4,542,102, and (1987) U.S. Pat. No. 4,713,326.

In another embodiment, colony or phage plaque transfer of biological polymers may be transferred directly onto a silicon substrate. For example, a colony plate may be transferred onto a substrate having a generic oligonucleotide sequence which hybridizes to another generic complementary sequence contained on all of the vectors into which inserts are cloned. This will specifically only bind those molecules which are actually contained in the vectors containing the desired complementary sequence. This immobilization allows for producing a matrix onto which a sequence specific reagent can bind, or for other purposes. In a further embodiment, a plurality of different vectors each having a specific oligonucleotide attached to the vector may be specifically attached to particular regions on a matrix having a complementary oligonucleotide attached thereto.

VIII. HYBRIDIZATION/SPECIFIC INTERACTION

A. General

As discussed previously in the VLSIPS™ Technology parent applications, the VLSIPS™ technology substrates may be used for screening for specific interactions with sequence specific targets or probes.

In addition, the availability of substrates having the entire repertoire of possible sequences of a defined length opens up the possibility of sequencing by hybridization. This sequence may be de novo determination of an unknown sequence, particularly of nucleic acid, verification of a sequence determined by another method, or an investigation of changes in a previously sequenced gene, locating and identifying specific changes. For example, often Maxam and Gilbert sequencing techniques are applied to sequences which have been determined by Sanger and Coulson. Each of those sequencing technologies have problems with resolving particular types of sequences. Sequencing by hybridization may serve as a third and independent method for verifying other sequencing techniques. See, e.g., (1988) *Science* 242:1245.

In addition, the ability to provide a large repertoire of particular sequences allows use of short subsequences and hybridization as a means to fingerprint a sample. This may be used in a nucleic acid, as well as other polymer embodiments. For example, fingerprinting to a high degree of specificity of sequence matching may be used for identifying highly similar samples, e.g., those exhibiting high homology to the selected probes. This may provide a means for determining classifications of particular sequences. This should allow determination of whether particular genomes of bacteria, phage, or even higher cells might be related to one another.

In addition, fingerprinting may be used to identify an individual source of biological sample. See, e.g., Lander, E. (1989) *Nature*, 339:501–505, and references therein. For example, a DNA fingerprint may be used to determine whether a genetic sample arose from another individual. This would be particularly useful in various sorts of forensic tests to determine, e.g., paternity or sources of blood samples. Significant detail on the particulars of genetic fingerprinting for identification purposes are described in, e.g., Morris et al. (1989) "Biostatistical evolution of evidence from continuous allele frequency distribution DNA probes in reference to disputed paternity of identity," *J. Forensic Science* 34:1311–1317; and Neufeld et al. (1990) *Scientific American* 262:46–53; each of which is hereby incorporated herein by reference.

In another embodiment, a fingerprinting-like procedure may be used for classifying cell types by analyzing a pattern

of specific nucleic acids present in the cell. A series of antibodies may be used to identify cell markers, e.g., proteins, usually on the cell surface, but intracellular markers may also be used. Antigens which are extracellularly expressed are preferred so cell lysis is unnecessary in the screening, but intracellular markers may also be useful. The markers will usually be proteins, but may be nucleic acids, lipids, metabolites, carbohydrates, or other cellular components. See, e.g., Winkelgren, I. (1990) *Science News 136:234–237*, which indicates extracellular DNA may be common, and suggesting that such might be characteristic of cell types, stage, or physiology. This may also be useful in defining the temporal stage of development of cells, e.g., stem cells or other cells which undergo temporal changes in development. For example, the stage of a cell, or group of cells, may be tested or defined by isolating a sample of mRNA from the population and testing to see what sequences are present in messenger populations. Direct samples, or amplified samples, may be used. Where particular mRNA or other nucleic acid sequences may be characteristic of or shown to be characteristic of particular developmental stages, physiological states, or other conditions, this fingerprinting method may define them. Similar sorts of fingerprinting may be used for determining T-cell classes or perhaps even to generate classification schemes for such proteins as major histocompatibility complex antigens. Thus, the ability to make these substrates allows both the generation of reagents which will be used for defining subclasses or classes of cells or other biological materials, but also provides the mechanisms for selecting those cells which may be found in defined population groups.

In addition to cell classification defined by such a combination of properties, typically expression of extracellular antigens, the present invention also provides the means for isolating homogeneous population of cells. Once the antigenic determinants which define a cell class have been identified, these antigens may be used in a sequential selection process to isolate only those cells which exhibit the combination of defining structural properties.

The present invention may also be used for mapping sequences within a larger segment. This may be performed by at least two methods, particularly in reference to nucleic acids. Often, enormous segments of DNA are subcloned into a large plurality of subsequences. Ordering these subsequences may be important in determining the overlaps of sequences upon nucleotide determinations. Mapping may be performed by immobilizing particularly large segments onto a matrix using the VLSIPS™ Technology. Alternatively, sequences may be ordered by virtue of subsequences shared by overlapping segments. See, e.g., Craig et al. (1990) *Nuc. Acids Res.* 18:2653–2660; Michiels et al. (1987) *CABIOS* 3:203–210; and Olson et al. (1986) *Proc. Natl. Acad. Sci. USA* 83:7826–7830.

B. Important Parameters

The extent of specific interaction between reagents immobilized to the VLSIPS™ Technology substrate and another sequence specific reagent may be modified by the conditions of the interaction. Sequencing embodiments typically require high fidelity hybridization and the ability to discriminate perfect matching from imperfect matching. Fingerprinting and mapping embodiments may be performed using less stringent conditions, depending upon the circumstances.

For example, the specificity of antibody/antigen interaction may depend upon such parameters as pH, salt concentration, ionic composition, solvent composition, detergent composition and concentration, and chaotropic agent concentration. See, e.g., Harlow and Lane (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Press, New York. By careful control of these parameters, the affinity of binding may be mapped across different sequences.

In a nucleic acid hybridization embodiment, the specificity and kinetics of hybridization have been described in detail by, e.g., Wetmur and Davidson (1968) *J. Mol. Biol.*, 31:349–370, Britten and Kohne (1968) *Science* 161:529–530, and Kanehisa, (1984) *Nuc. Acids Res.* 12:203–213, each of which is hereby incorporated herein by reference. Parameters which are well known to affect specificity and kinetics of reaction include salt conditions, ionic composition of the solvent, hybridization temperature, length of oligonucleotide matching sequences, guanine and cytosine (GC) content, presence of hybridization accelerators, pH, specific bases found in the matching sequences, solvent conditions, and addition of organic solvents.

In particular, the salt conditions required for driving highly mismatched sequences to completion typically include a high salt concentration. The typical salt used is sodium chloride (NaCl), however, other ionic salts may be utilized, e.g., KCl. Depending on the desired stringency hybridization, the salt concentration will often be less than about 3 molar, more often less than 2.5 molar, usually less than about 2 molar, and more usually less than about 1.5 molar. For applications directed towards higher stringency matching, the salt concentrations would typically be lower. Ordinary high stringency conditions will utilize salt concentration of less than about 1 molar, more often less then about 750 millimolar, usually less than about 500 millimolar, and may be as low as about 250 or 150 millimolar.

The kinetics of hybridization and the stringency of hybridization both depend upon the temperature at which the hybridization is performed and the temperature at which the washing steps are performed. Temperatures at which steps for low stringency hybridization are desired would typically be lower temperatures, e.g., ordinarily at least about 15° C., more ordinarily at least about 20° C., usually at least about 25° C., and more usually at least about 30° C. For those applications requiring high stringency hybridization, or fidelity of hybridization and sequence matching, temperatures at which hybridization and washing steps are performed would typically be high. For example, temperatures in excess of about 35° C. would often be used, more often in excess of about 40° C., usually at least about 45° C., and occasionally even temperatures as high as about 50° C. or 60° C. or more. Of course, the hybridization of oligonucleotides may be disrupted by even higher temperatures. Thus, for stripping of targets from substrates, as discussed below, temperatures as high as 80° C., or even higher may be used.

The base composition of the specific oligonucleotides involved in hybridization affects the temperature of melting, and the stability of hybridization as discussed in the above references. However, the bias of GC rich sequences to hybridize faster and retain stability at higher temperatures can be compensated for by the inclusion in the hybridization incubation or wash steps of various buffers. Sample buffers which accomplish this result include the triethly-and trimethyl ammonium buffers. See, e.g., Wood et al. (1987) *Proc. Natl. Acad. Sci. USA*, 82:1585–1588, and Khrapko, K. et al. (1989) *FEBS Letters* 256:118–122.

The rate of hybridization can also be affected by the inclusion of particular hybridization accelerators. These hybridization accelerators include the volume exclusion agents characterized by dextran sulfate, or polyethylene glycol (PEG). Dextran sulfate is typically included at a

concentration of between 1% and 40% by weight. The actual concentration selected depends upon the application, but typically a faster hybridization is desired in which the concentration is optimized for the system in question. Dextran sulfate is often included at a concentration of between 0.5% and 2% by weight or dextran sulfate at a concentration between about 0.5% and 5%. Alternatively, proteins which accelerate hybridization may be added, e.g., the recA protein found in *E. coli* or other homologous proteins.

With respect to those embodiments where specific reagents are not oligonucleotides, the conditions of specific interaction would depend on the affinity of binding between the specific reagent and its target. Typically parameters which would be of particular importance would be pH, salt concentration anion and cation compositions, buffer concentration, organic solvent inclusion, detergent concentration, and inclusion of such reagents such as chaotropic agents. In particular, the affinity of binding may be tested over a variety of conditions by multiple washes and repeat scans or by using reagents with differences in binding affinity to determine which reagents bind or do not bind under the selected binding and washing conditions. The spectrum of binding affinities may provide an additional dimension of information which may be very useful in identification purposes and mapping.

Of course, the specific hybridization conditions will be selected to correspond to a discriminatory condition which provides a positive signal where desired but fails to show a positive signal at affinities where interaction is not desired. This may be determined by a number of titration steps or with a number of controls which will be run during the hybridization and/or washing steps to determine at what point the hybridization conditions have reached the stage of desired specificity.

IX. DETECTION METHODS

Methods for detection depend upon the label selected. The criteria for selecting an appropriate label are discussed below, however, a fluorescent label is preferred because of its extreme sensitivity and simplicity. Standard labeling procedures are used to determine the positions where interactions between a sequence and a reagent take place. For example, if a target sequence is labeled and exposed to a matrix of different probes, only those locations where probes do interact with the target will exhibit any signal. Alternatively, other methods may be used to scan the matrix to determine where interaction takes place. Of course, the spectrum of interactions may be determined in a temporal manner by repeated scans of interactions which occur at each of a multiplicity of conditions. However, instead of testing each individual interaction separately, a multiplicity of sequence interactions may be simultaneously determined on a matrix.

A. Labeling Technicues

The target polynucleotide may be labeled by any of a number of convenient detectable markers. A fluorescent label is preferred because it provides a very strong signal with low background. It is also optically detectable at high resolution and sensitivity through a quick scanning procedure. Other potential labeling moieties include, radioisotopes, chemiluminescent compounds, labeled binding proteins, heavy metal atoms, spectroscopic markers, magnetic labels, and linked enzymes.

Another method for labeling may bypass any label of the target sequence. The target may be exposed to the probes, and a double strand hybrid is formed at those positions only. Addition of a double strand specific reagent will detect where hybridization takes place. An intercalative dye such as ethidium bromide may be used as long as the probes themselves do not fold back on themselves to a significant extent forming hairpin loops. See, e.g., Sheldon et al. (1986) U.S. Pat. No. 4,582,789. However, the length of the hairpin loops in short oligonucleotide probes would typically be insufficient to form a stable duplex.

In another embodiment, different targets may be simultaneously sequenced where each target has a different label. For instance, one target could have a green fluorescent label and a second target could have a red fluorescent label. The scanning step will distinguish sites of binding of the red label from those binding the green fluorescent label. Each sequence can be analyzed independently from one another.

Suitable chromogens will include molecules and compounds which absorb light in a distinctive range of wavelengths so that a color may be observed, or emit light when irradiated with radiation of a particular wave length or wave length range, e.g., fluorescers. Biliproteins, e.g., phycoerythrin, may also serve as labels.

A wide variety of suitable dyes are available, being primarily chosen to provide an intense color with minimal absorption by their surroundings. Illustrative dye types include quinoline dyes, triarylmethane dyes, acridine dyes, alizarine dyes, phthaleins, insect dyes, azo dyes, anthraquinoid dyes, cyanine dyes, phenazathionium dyes, and phenazoxonium dyes.

A wide variety of fluorescers may be employed either by themselves or in conjunction with quencher molecules. Fluorescers of interest fall into a variety of categories having certain primary functionalities. These primary functionalities include 1- and 2-aminonaphthalene, p,p'-diaminostilbenes, pyrenes, quaternary phenanthridine salts, 9-aminoacridines, p,p'-diaminobenzophenone imines, anthracenes, oxacarbocyanine, merocyanine, 3-aminoequilenin, perylene, bis-benzoxazole, bis-p-oxazolyl benzene, 1,2-benzophenazin, retinol, bis-3-aminopyridinium salts, hellebrigenin, tetracycline, sterophenol, benzimidzaolylphenylamine, 2-oxo-3-chromen, indole, xanthen, 7-hydroxycoumarin, phenoxazine, salicylate, strophanthidin, porphyrins, triarylmethanes and flavin. Individual fluorescent compounds which have functionalities for linking or which can be modified to incorporate such functionalities include, e.g., dansyl chloride; fluoresceins such as 3,6-dihydroxy-9-phenylxanthhydrol; rhodamineisothiocyanate; N-phenyl 1-amino-8-sulfonatonaphthalene; N-phenyl 2-amino-6-sulfonatonaphthalene; 4-acetamido-4-isothiocyanatostilbene-2,2'-disulfonic acid; pyrene-3-sulfonic acid; 2-toluidinonaphthalene-6-sulfonate; N-phenyl, N-methyl 2-aminoaphthalene-6-sulfonate; ethidium bromide; stebrine; auromine-0,2-(9'-anthroyl)palmitate; dansyl phosphatidylethanolamine; N,N'-dioctadecyl oxacarbocyanine; N,N'-dihexyl oxacarbocyanine; merocyanine, 4-(3'pyrenyl) butyrate; d-3-aminodesoxy-equilenin; 12-(9'-anthroyl) stearate; 2-methylanthracene; 9-vinylanthracene; 2,2'-(vinylene-p-phenylene)bisbenzoxazole; p-bis[2-(4-methyl-5-phenyl-oxazolyl)]benzene; 6-dimethylamino-1,2-benzophenazin; retinol; bis(3'-aminopyridinium) 1,10-decandiyl diiodide; sulfonaphthylhydrazone of hellibrienin; chlorotetracycline; N-(7-dimethylamino-4-methyl-2-oxo-3-chromenyl)maleimide; N-[p-(2-benzimidazolyl)-phenyl] maleimide; N-(4-fluoranthyl)maleimide; bis(homovanillic acid); resazarin; 4-chloro-7-nitro-2,1,3-benzooxadiazole; merocyanine 540; resorufin; rose bengal; and 2,4-diphenyl-3(2H)-furanone.

Desirably, fluorescers should absorb light above about 300 nm, preferably about 350 nm, and more preferably